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Published in:
Journal of Cellular Physiology

DOI:
10.1002/jcp.29901

Publication date:
2021

Document Version
Publisher's PDF, also known as Version of record

Received: 5 February 2020 | Accepted: 13 June 2020

DOI: 10.1002/jcp.29901

ORIGINAL RESEARCH ARTICLE

Journal of Cellular Physiology WILEY

Cx31.1 expression is modulated in HaCaT cells exposed to UV-induced damage and scrape-wounding

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Funding information
Medical Research Scotland,
Grant/Award Number: Vac-704-2013

Abstract

Connexin31.1 (Cx31.1) is a gap junction protein associated with apoptosis. In the skin, apoptosis is modulated by diabetes. A HaCaT skin model investigated whether normal (NGI) and high glucose and insulin (HGI; diabetic) conditions altered Cx31.1 expression, and if these were apoptosis linked. Cx31.1 was found in HaCaT and HeLa Ohio cells, with HaCaT Cx31.1 protein increased in HGI conditions, and around apoptotic cells. HeLa Cx31.1 channels were noncommunicative. Post scrape-wounding, Cx31.1 increased at wound edges. Caspase 3/7 in scrape-wounds media (containing cells) elevated in HGI. UV exposure raised Cx31.1, and caspase 3/7, in NGI and HGI. UV reduced cell viability in NGI cells, although not significantly in HGI. Cx31.1 is modulated during HaCaT cell wound closure, and associated with 'diabetic' conditions. Cx31.1 expression matched apoptosis levels, higher in HGI cultures. Cx31.1 is noncommunicating, modulated after wounding, linked to apoptosis, and may be associated with tissue turn-over around diabetic wounds.

KEYWORDS

apoptosis, Cx31.1, diabetes, gap junctions, wound healing

1 | INTRODUCTION

In diabetes, wound healing is compromised and wounds often fail to close, resulting in diabetic ulcers which are difficult to heal and costly to treat. Gap junction expression is well-documented to be compromised in diabetic skin and chronic wounds, and failure of gap junction intercellular communication, essential in coordinating wound healing, is a contributing factor to ulcer formation (Becker, Thrasivoulou, & Phillips, 2012; Martin, Easton, Hodgins, & Wright, 2014; J. A. Wright, Richards, & Becker, 2012). Connexins are a family of transmembrane proteins that assemble to form connexons or hemichannels in the plasma membrane. Pairs of connexon channels dock making gap junctions, which directly link the cytoplasm of adjoining cells, allowing them to communicate. Inorganic ions, small metabolites, and cellular signalling molecules (<1 kDa in size) are exchanged between cells via these gap junctions (Evans & Martin, 2002; Laird, 2006). This allows for cells within a tissue to coordinate their processes, and they are important in many organs such as the heart, brain and skin.

In the skin, the epidermis is avascular and gap junction communication is important in epidermal barrier integrity, epidermal stem cell function, and keratinocyte terminal differentiation pathways (Mascre et al., 2012). Up to 10 connexin subtypes are differentially expressed in the skin in various layers with connexin43 (Cx43) predominating: 9 connexins in the epidermis and 3 in the dermis (Martin et al., 2014). Cx31.1 is expressed in the epidermal spinous and granular layers (Di, Rugg, Leigh, & Kelsell, 2001; Gibson, Bikle, Harris, & Goldberg, 1997; Hennemann et al., 1992). Connexin expression

levels in HaCaT cells (a human keratinocyte cell line) are similar to those found in human keratinocytes and they are a frequently used model for epidermal skin cells (Boukamp et al., 1988). During skin wound healing, distribution patterns of Cx43 and Cx26 are remodelled, and Cx43 is a therapeutic target to improve wound closure (reviewed by Becker et al., 2012). In diabetic wound healing, Cx43 and Cx26 levels do not change as expected, which may be related to wound closure failure. Alongside these major players, reports show that Cx30, Cx31, and Cx31.1 can also be dynamically regulated during wound repair (Goliger & Paul, 1995).

Cx31.1 is a less well-studied connexin, but appears to have roles in cell and tissue turn-over. In the eye (Chang et al., 2009) and ovary (Wright, Becker, Lin, Warner, & Hardy, 2001), its expression is associated with apoptosis, and it is thought to form gap junctions which are nonfunctional and not communicative. The role of Cx31.1 in skin function and wound healing is not defined, however, and tissue turnover is compromised around diabetic wound edges, where epidermal hyperproliferation occurs. Cx31.1 may be involved in this aberrant tissue turnover. Different mechanisms can affect cell proliferation and/or migration during wound closure, and prevent it. In diabetic wounds, one of these factors could be regulation and expression of Cx31.1.

This study investigated whether Cx31.1 expression was altered in HaCaT scrape-wounded monolayers cultured in normal (NGI) and high glucose and insulin (HGI) and if this was associated with UV-induced apoptosis. Culture conditions mimicking the environment cells would be exposed to in normal and untreated type II diabetic conditions, where high circulating levels of glucose and insulin occur. We propose that levels of Cx31.1 and apoptosis are different in normal and diabetic skin following wounding, and are thus contributing factors in delayed diabetic wound healing.

2 | MATERIALS AND METHODS

2.1 | Cell sources and culture

HaCaT and HeLa Ohio cells were obtained from Cell Lines Services (Eppelhiem, Germany) and the European Collection of Authenticated Cell Cultures (via Merck/Sigma Aldrich, Gillingham, UK), respectively. Human dermal neonatal fibroblast cells were obtained from Invitrogen (Paisley, UK). Cells were maintained in euglycaemic Dulbecco's modified Eagle's medium (DMEM) containing 5.5 mM glucose supplemented with 10% vol/vol fetal bovine serum, 2 mM glutamine, 50 U/ml penicillin, and 50 U/ml streptomycin (all Lonza, Wokingham, UK). This complete media was known as cDMEM. To mimic 'normal' and 'type II diabetic' levels of glucose and insulin in the skin, following plating, HaCaT cells were treated daily for a minimum of 5 days with 5.5 mM glucose, 1 nM insulin and 0.1% bovine serum albumin (BSA; normal glucose and insulin [NGI] conditions), or 25 mM glucose, 10 nM insulin, and 0.1% BSA (high glucose and insulin [pHG] conditions) in DMEM before experiments being carried out (Bagdade, Bierman, & Porte, 1967; Spravchikov et al., 2001;Wright, Berends, Flint, & Martin, 2013). This regime allowed preconditioning of the cells to 'normal' and 'diabetic' cellular environments, and has been shown by other studies to change gene expression, for example, Cx43 increases (Wright et al., 2013), such that a diabetic phenotype is mimicked by the HGI treatment, which is similar to that seen in primary skin cells from patients with diabetes Levels of insulin and glucose used here represent that seen in the skin of type II diabetic patients who are not receiving treatment (Bagdade et al., 1967).

2.2 | Ultra violet exposure of HaCaT cells

For induction of apoptosis in some HaCaTs, cells were grown to confluence in a 96-well plate and pretreated with NGI and HGI as above. Culture medium was removed from the cells, replaced by phosphate buffered saline (PBS), and they were exposed to 4,000 μW/cm^2 ultra violet (UV) light in a UV Crosslinker (Thermo Fisher Scientific, Loughborough, UK) for 1 min. Culture medium was replaced, the cells returned to the incubator, and the relevant assay carried out 18 hr later. For the control group, the media in dishes were replaced with PBS as above, but half of the plate was then covered and only the other half of the plate was exposed to the UV light. The media were then replenished and the dishes returned to the incubator.

2.3 | Western blot analysis

To test whether Cx31.1 was expressed in HeLa Ohio cells, western blot analysis was carried out. Cells (1×10^6) were plated onto six-well plates, grown to subconfluence, and harvested. Western blot analysis was performed as in (Easton, Petersen, & Martin, 2009). The primary antibody was a rabbit polyclonal Cx31.1 antibody (1:1,000; custom made for Prof. Colin Green, University of Auckland, New Zealand) and a rabbit polyclonal glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody (diluted 1:10,000; Abcam, Cambridge, UK). Blots were developed using Uptilight™ High WB Chemiluminescent Substrate (Cheshire Sciences [UK] Ltd, Chester, UK) and analysed via a LI-COR Odyssey system (LI-COR, Cambridge, UK).

2.4 | Gap junction functionality assays

To assess whether there were functional gap junctions in Hela Ohio cells, scrape load assays were first performed. Cells were grown to confluence in a 60 mm dish, washed in PBS, and then scraped with a 200 μl pipette tip. A 1:200 dilution of Alexa Flour 488 or Alexa Flour 594 dye (equivalent to 5 μg/ml; Invitrogen, Paisley, UK) was added and left for 5 min. The cells were washed in PBS and fixed with 3.7% paraformaldehyde. After 5 min, the cells were washed in PBS. Images were then taken with a LSM800 confocal microscope (Zeiss, Cambridge, UK).

Gap junction functionality was further assessed by parachute assay (Stains & Civitelli, 2016). HeLa Ohio or HeLa43 cells (HeLa cells stably transfected with Cx43) were seeded onto cover-chambers and

six-well plates, respectively, at 1×10^6 cells per well. Briefly, cells were live-stained with 2.5 mM calcein-AM and layered at a ratio of 1:50 onto cells live-stained with 1 mg/ml CM-DiI (Invitrogen) and incubated at 37°C for 4 hr. Some HeLa43 cells were treated with 100 μM carbenoxolone to block gap junctional intercellular communication (GJIC). Dye transfer was assessed using confocal microscopy by detecting dye spread from the green-stained donor cells into the red-stained acceptor cells, the average percentage of cells receiving calcein-AM being calculated (Martin, Wall, & Griffith, 2005).

2.5 | Quantitative real-time polymerase chain reaction

HaCaTs were plated onto six-well plates (1×10^6 cells/well), grown to confluence and preconditioned with NGI or HGI for 5 days. mRNA was extracted with an RNeasy plus mini kit (Qiagen, Crawley, UK), and quality assessed with a Nanodrop (Thermo Fisher Scientific). Reverse transcription was carried out on 1 μg mRNA per reaction with reverse transcriptase (Roche, Welwyn Garden City, UK) according to the manufacturer's instructions. Taqman-type qPCR was carried out using primers and probes to human Cx31.1 in comparison with human GAPDH as a control.

Human Cx31.1 qPCR primers and probes (IDT, Leuven, Belgium) were forward primer: TGTGTGGAGTGATGACCACAAGGA, reverse primer: ACATGGGACACAGGGAAGAACTCA and probe: CCGGCT GCTCCAACGTCTGCTTTGA. Human GAPDH primers and probes were forward primer: CACATGGCCTCCAAGGAGTAA, reverse primer: TGAGGGTCTCTCTCTTCCTCTTGT, and probe: CTGGACCA CCAGCCCCAGCAAG.

2.6 | Scrape-wound assays and immunocytochemistry

HaCaTs were seeded on glass coverslips at 1×10^6/ml in 12-well plates, and grown to confluent monolayers in NGI or HGI. After treatment, scrape-wounds were introduced with a 100 μl pipette tip and cell migration monitored (Wright, van Steensel, Hodgins, & Martin, 2009). Cells were fixed at 0, 24, and 48 hr with 100% ice cold methanol. Immunocytochemistry was carried out as described previously (Wright et al., 2009). Anti-rabbit polyclonal primary antibodies diluted with filtered PBS were used: anti-Cx31.1 was diluted 1:500. Anti-active caspase 3 (Promega, Southampton, UK) was used at 1:250. Secondary antibodies were goat anti-rabbit Alexa Flour 488 (diluted 1:500; equivalent to 2 μg/ml; Invitrogen) and Alexa Flour 594 (1:500; Invitrogen) for Cx31.1 and active caspase 3, respectively. Cell nuclei were labelled with 10 μg/ml 4′,6-diamidino-2-phenylindole (Invitrogen) and images taken via confocal microscopy (Wright et al., 2009). Similar immunocytochemistry for Cx31.1 expression detection was carried out on HeLa Ohio cells. Pixel intensity analysis was carried out via the ImageJ software (NIH) on three independent images for each condition.

2.7 | Caspase 3/7 assays

Caspase 3/7 activity was either measured in NGI- or HGI-treated HaCaTs (5×10^4 cells/ml) in 96-well plates, or in conditioned media which contained free-floating apoptotic cells. Caspase 3/7 was measured using the Caspase-Glo 3/7 kit (Promega) and luminescence was measured on a Flourstar Optima microplate reader (BMG Labtech, Aylesbury, UK).

2.8 | Cell viability assay

HaCaT cell viability was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) absorbance assay. About 5 mg/ml of MTT stock (Fisher) was added to NGI or HGI-treated cells plated at 5×10^4 cells/ml in a 96 well plate and incubated for 1 hr. Dimethyl sulfoxide was added to solubilise the cells, and the plate was read for absorbance at 570 nm on a Floustar Optima microplate reader (BMG Labtech, Aylesbury, UK).

2.9 | Statistical analysis

All experiments were done a minimum of three times ($n \leq 3$). Normalisation of data was carried out in Excel. Results were compiled into the GraphPad Prism software (GraphPad Software Inc, La Jolla, CA) and all data is expressed as mean ± standard error of mean. Statistical analysis was performed on the data using a paired *t* test with statistical significance inferred at $p < .05$ for all experiments.

3 | RESULTS

3.1 | Cx31.1 is expressed in HeLa Ohio and HaCaT cell membranes

HeLa Ohio cells have been reported not to express any other connexin subtypes at appreciable mRNA or protein levels (Eckert, Dunina-Barkovskaya, & Hulser, 1993; Mesnil et al., 1995). They are often used as a vehicle for connexin transfection for this reason. To determine whether Cx31.1 specifically was expressed in HeLa Ohio, HaCaT and neonatal dermal fibroblast cells, and western blot analysis was carried out (Figure 1a). This revealed that Cx31.1 protein was expressed in HeLa Ohio cells and in HaCaT cells, although not in dermal fibroblasts. Cx31.1 was not previously known to be expressed in HeLa Ohio cells, so this is a novel result.

3.2 | HeLa Ohio cells do not transfer dye between them

To test whether dye transferred through cell layers, indicating GJIC, HeLa Ohio cells were scrape-loaded with Alexa Flour dye. This

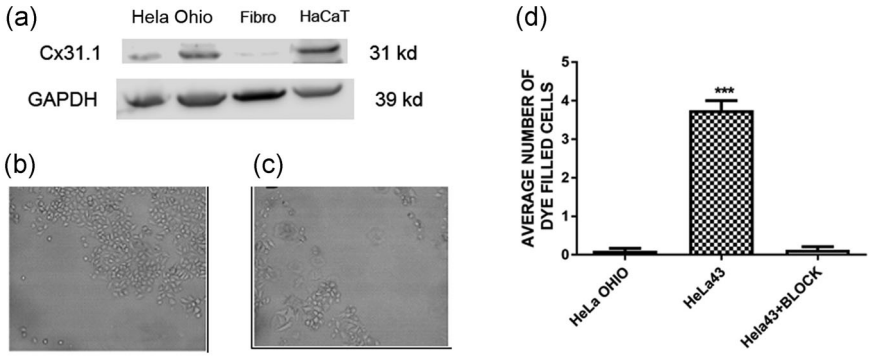

FIGURE 1 (a) Western blot analysis for Cx31.1 in HeLa Ohio, dermal fibroblasts, and HaCaT cells, showed Cx31.1 protein expression in all cell types apart from dermal fibroblasts (negative control). (b) Scrape load assays with HeLa Ohio cells exposed to Alexa Flour 488 (1:500), revealed no appreciable uptake of dye or dye transfer between the cell layers in cells scraped and PBS added, or where Alexa 488 was added (c). (d) Calcein-AM parachute assays showed that HeLa Ohio cells did not transfer dye from donor to acceptor cells via GJIC, whereas in HeLa43 cells dye was transferred to four cells on average (***$p < .001$). HeLa43 cells blocked with 100 μM carbenoxolone exhibited similar levels of gap junctional coupling as HeLa Ohio cells alone ($n = 3$, paired t test). GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GJIC, gap junctional intercellular communication; PBS, phosphate buffered saline

indicated whether the low levels of Cx31.1 expressed by HeLa Ohio cells were able to form functional communicating channels. There was no significant dye transfer between the cells (Figure 1b,c), thus GJIC was not active, confirming previous data. In further dye transfer studies (Figure 1d), a parachute assay showed that donor HeLa Ohio cells do not transfer Calcein-AM dye to acceptor cells, whereas in HeLa43 cells, dye was transferred to four cells on an average (Figure 1d). HeLa43 cells blocked with 100 μM carbenoxolone exhibited similar levels of transfer to HeLa Ohio cells alone. We can therefore confirm that in HeLa Ohio cells, Cx31.1 does not form functional gap junctions, and this can be extrapolated to HaCaT cells.

3.3 | Cx31.1 expression is raised in 'diabetic' conditions and after UV exposure

HaCaT cells were treated with NGI or HGI media for 5 days to simulate normal and 'diabetic' conditions. Five days of pretreatment to cells in this way has previously been shown to induce gene expression (Wright et al., 2013) and to mimic diabetic-induced changes to connexins and other proteins (Pollok et al., 2011; Spravchikov et al., 2001; Wright, Pollok, Flint, Brandner, & Martin, 2012). qPCR of Cx31.1 gene expression was performed and normalised to GAPDH expression. This showed that there was a small (though not statistically significant) increase in Cx31.1 mRNA expression in HGI compared to NGI treatment (Figure 2a).

Immunocytochemistry revealed Cx31.1 protein expression (green punctate staining) on cell membranes in areas of cell-cell contact and on membranes without contact as well (Figure 2b,c). There was also some cytoplasmic labelling of Cx31.1 in HGI-treated cells apparent as intracellular speckles. HaCaTs treated with HGI showed higher levels of expression of Cx31.1 protein compared to those treated with NGI, and this was confirmed by pixel intensity analysis (Figure 2f). In addition, Cx31.1 was particularly apparent around apoptotic cells where often nuclei had broken down. Figure 2d shows Cx31.1 differentially expressed around an apoptotic cell, as defined by nuclear shape, exhibiting higher levels of membrane Cx31.1 compared to the normal cells around it. HeLa Ohio cells showed a low level of Cx31.1 expression on the cell membranes. This appeared to be on healthy cells as well as those undergoing cell death.

3.4 | UV exposure of human skin is a key mediator of apoptosis

To determine if apoptosis of HaCaT cells that was initiated by UV exposure (as would occur to epidermal keratinocytes in human skin on sun exposure) would affect Cx31.1 expression, cells were cultured in NGI or HGI, exposed to UV, and then underwent immunocytochemistry. Figure 2e shows that Cx31.1 expression was increased in both NGI- and HGI-treated cells by UV exposure. This was confirmed by pixel intensity analysis of the Cx31.1 expression which showed a nonsignificant increase after UV treatment for NGI treated cells, but a significant rise for HGI treated cells ($p < .05$; Figure 2f).

3.5 | Cx31.1 and apoptosis are modulated during scrape-wounding

The HaCaTs were scrape-wounded and Cx31.1 expression examined post-wounding (Figure 3a). Cells migrated over the denuded area over the next 48 hr. In comparison with just after wounding (0 hr), Cx31.1 expression at wound edges is increased at 24 and 48 hr in both NGI- and HGI-treated cells, with higher levels of Cx31.1 in HGI conditions compared to NGI conditions at all time-points. In addition, there was greater intracellular speckling of Cx31.1 within the

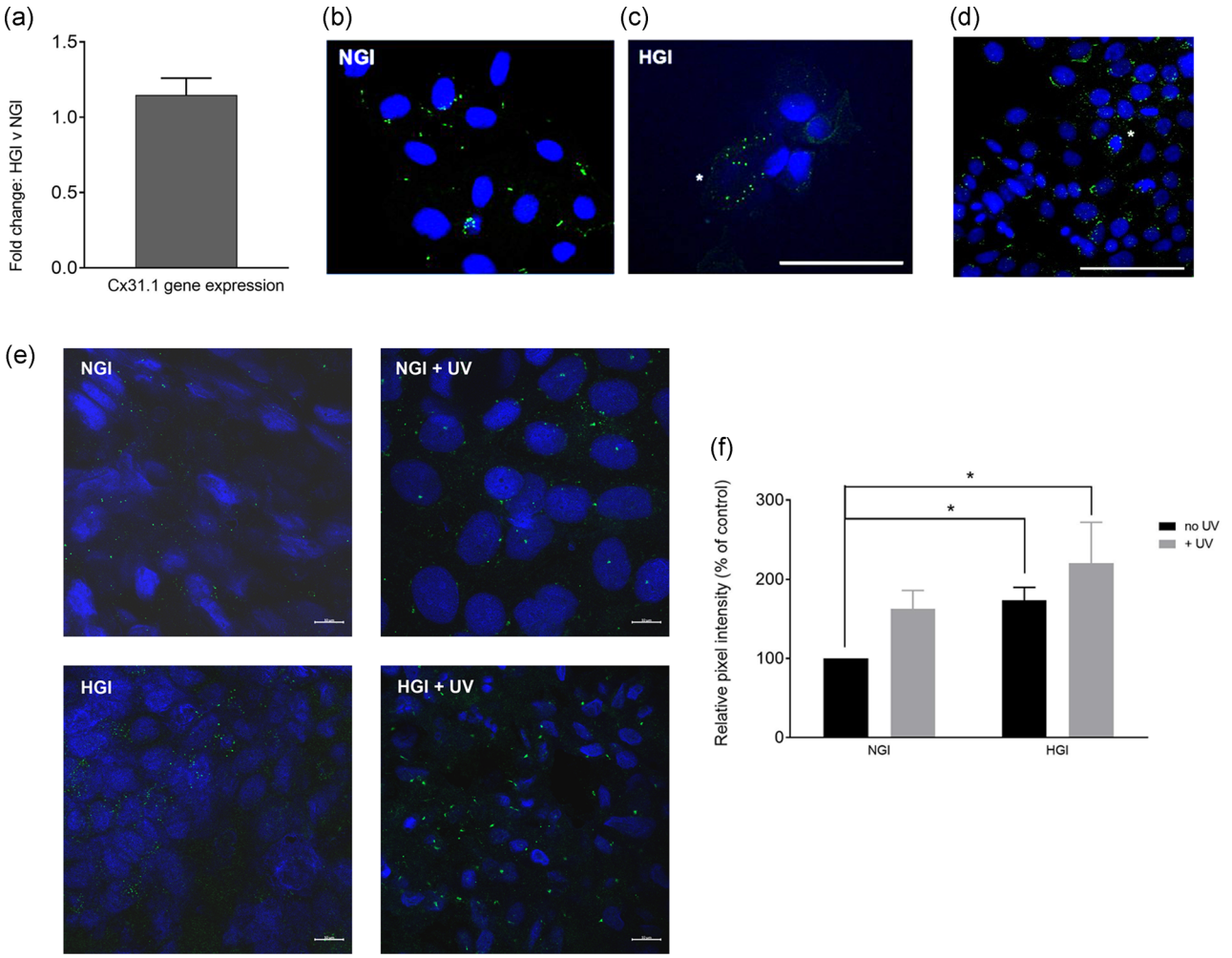

FIGURE 2 (a) Fold change of Cx31.1 expression via qPCR in HaCaTs treated with HGI versus NGI normalised to GAPDH. There was is a nonstatically significant increase in HGI conditions compared to NGI on Cx31.1 mRNA ($n = 5$). (b,c) Immunocytochemistry of Cx31.1 (green punctate staining) in HaCaTs with DAPI stained nuclei (blue) showed Cx31.1 was present on cell membranes, with some cytoplasmic labelling in HGI-treated cells. HaCaTs treated with HGI showed higher levels of Cx31.1 expression compared to those treated with NGI. (d) In untreated cells cultured in cDMEM Cx31.1 was apparent at a higher level around apoptotic cells (⋆), as defined by nuclear shape, where often nuclei had broken down, compared to healthy cells. (e) Expression of Cx31.1 in HaCaTs cultured in normal and 'diabetic' conditions pre- and postexposure to UV, that stimulated apoptosis pathways. UV exposure increased the levels of Cx31.1 in both NGI and HGI treated cells. (f) Relative pixel intensity measurement of Cx31.1 expression by immunocytochemistry confirmed an increase ($p < .05$) in Cx31.1 with both NGI and UV treatment ($n = 3$). Scale bars = 100 μm (c,d) or 10 μm (e). DAPI, 4′,6-diamidino-2-phenylindole; DMEM, Dulbecco's modified Eagle's medium; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HGI, high glucose and insulin; NGI, normal glucose and insulin; qPCR, qualitative polymerase chain reaction

cytoplasm of HGI cells—suggestive of internalised Cx31.1. To examine levels of apoptosis in scrape-wounded HaCaTs, we examined active caspase 3 which should only be expressed in cells undergoing apoptosis (Figure 3b). Active caspase 3 expression increased 24 hr after scrape-wounding in both NGI- and HGI-treated HaCaTs. This resolved by 48 hr postwounding. Thus there is some correlation between Cx31.1 and caspase 3/7 expression at the wound margins.

Caspase 3/7 activity (indicating levels of apoptosis) was measured in conditioned media containing apoptotic HaCaT cells obtained over the course of scrape-wound closure (Figure 4a). At 0 and 24 hr following the scrape-wound, the HGI media+cells contained higher levels of caspase activity than NGI media+cells ($p < .05$). However, this difference resolved at 48 hr. Caspase 3/7 is late marker of apoptosis, and caspase 3 is a common end point of the caspase assay cascade occurring after UV damage via death receptor activation, reactive oxygen species activation, and Bax translocation (C. H. Lee, Wu, Hong, Yu, & Wei, 2013).

3.6 | UV treatment increases apoptosis and decreases cell viability

To examine differences in susceptibility to apoptosis of HaCaT cells in normal and 'diabetic' conditions, some cells were exposed to UV

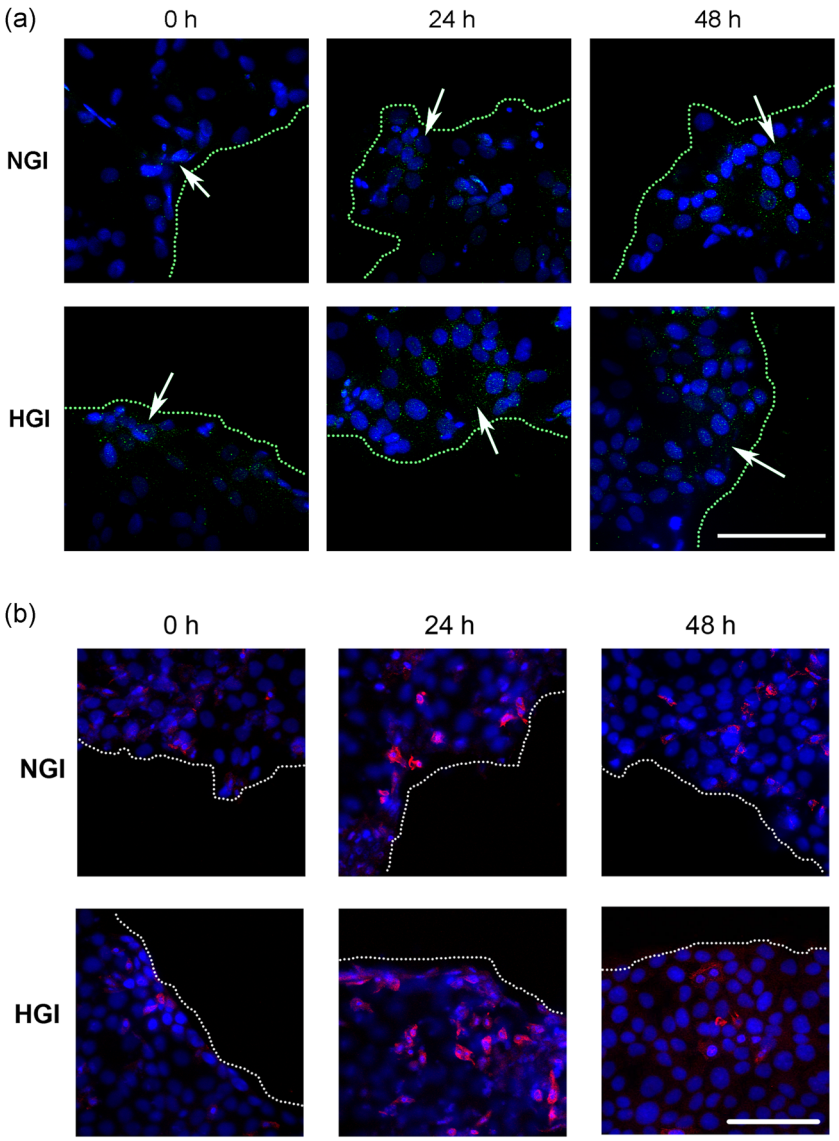

FIGURE 3 (a) Immunocytochemistry of Cx31.1 following scrape-wounding of HaCaTs. Dotted lines show the wound edges. Cx31.1 at wound margins (green punctate staining, indicated by arrows) increase at 24 and 48 hr compared to 0 hr in both NGI and HGI, with higher levels of Cx31.1 in HGI conditions compared to NGI conditions. (b) Immunocytochemistry of active caspase 3 indicating apoptosis cascade activation following scrape-wounding of HaCaTs. Active Caspase 3 (red staining) at wound edges was increased at 24 hr in both NGI and HGI conditions, indicating that apoptosis levels increased to 24 hr and then resolved. Scale bars = 100 μm. HGI, high glucose and insulin; NGI, normal glucose and insulin

(Figure 4b). UV exposure significantly increased caspase 3/7 activity in both NGI- ($p < .001$) and HGI-treated HaCaT cells ($p < .05$). However, HGI-treated cells showed reduced caspase activity compared to NGI-treated cells before UV exposure ($p < .01$).

In parallel to the apoptosis assays, the MTT cell viability assays determined there was reduced cell viability following UV exposure in NGI-treated cells compared to cells tested before UV exposure ($p < .001$). This did not occur in HGI-treated cells, although there was a similar trend (Figure 4c).

4 | DISCUSSION

Cx31.1 is a connexin subtype associated with apoptosis in organs such as the eye and ovary (Chang et al., 2009; Wright et al., 2001); however, it is not highly expressed in many tissues in normal conditions. It has also been unclear whether Cx31.1 makes functional communicating channels, and so if its expression may indicate a specific cell signalling route, or if blocks cell signalling, by closing down (Harris, 2001). In this study, we examined Cx31.1 expression in HaCaT cell model of the human epidermis, to investigate whether induction of apoptosis by UV exposure and culture in 'normal' and 'diabetic' skin conditions would influence Cx31.1 expression. Furthermore, we used HeLa Ohio cells to determine if Cx31.1 channels were open and functional.

We discovered that Cx31.1 protein was expressed at low levels in HeLa Ohio cell membranes (Figure 1), although it does not form communicating gap junctions when assessed by dye transfer assays. This fits similar studies where Cx31.1 has been found to be non-communicative or at least rarely forms functional gap junction channels either with itself or other connexin isoforms (Harris, 2001). We then investigated possible perturbations in Cx31.1 expression before and during scrape-wound healing. Cx31.1 was found to be increased in HaCaT cell membranes exposed to high levels of glucose

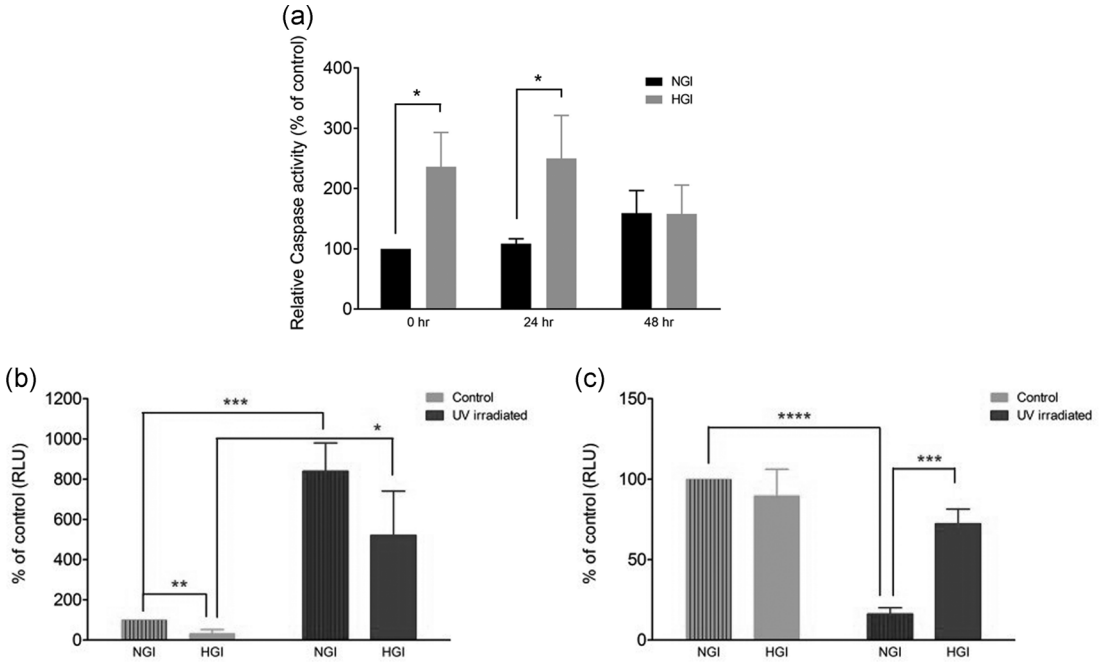

FIGURE 4 (a) Caspase 3/7 activity was measured in conditioned media containing apoptotic cells over the course of scrape-wound closure, and the data normalised. At 0 and 24 hr, HGI media+cells exhibited higher levels of caspase activity than NGI media+cells (*$p < .05$) during scrape-wound closure, but this resolved within 48 hr ($n > 3$; paired t test). (b) Caspase 3/7 apoptosis assays pre and post-18 hr UV exposure. Cellular caspase 3/7 activity revealed that UV exposure increased HaCaT apoptosis in NGI (***$p < .001$) and HGI (*$p < .05$) to a lesser extent compared to cells not exposed to UV. (c) The MTT assay indicated that cell viability decreased in NGI (****$p < .001$), but not significantly in HGI conditions, post-UV exposure ($n \geq 3$; paired t test). HGI, high glucose and insulin; NGI, normal glucose and insulin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

and insulin (mimicking those seen in the skin of people with uncontrolled type II diabetes) compared to normal levels (Figure 2b,c). Cx31.1 levels were also higher on membranes of apoptotic rather than viable cells. In addition, there was greater intracellular "speckling" of Cx31.1 in cells cultured with HGI, which may represent cleaved or internalised Cx31.1, although Cx31.1 does not have caspase 3/7 cleavage sites and cleavage was not seen in western blots. mRNA shows little change with HGI treatment (Figure 2a), thus any differences seen in Cx31.1 protein expression are likely to be posttranslational via high glucose levels increasing connexin phosphorylation.

The increase in HaCaT Cx31.1 in 'diabetic' culture conditions mirrors that seen in Cx43 and Cx26 exposed to high glucose and insulin conditions in diabetic skin, suggesting that there is a commonality in effects of diabetic conditions on connexins (Becker et al., 2012). Mechanisms that affect Cx43 in diabetic wound healing are overexpression and reduction of channel function via phosphorylation (Solan & Lampe, 2014), leading to a fall in GJIC (Abdullah et al., 1999; J. A. Wright et al., 2012). High levels of glucose and insulin deleteriously affect Cx43-containing gap junctions and hemichannels in human dermal fibroblasts (Wright et al., 2012) and keratinocytes where HGI conditions increased phosphorylation of Cx43 at Ser368 leading to a reduced function (Wright et al., 2013). Cx31.1 may also become phosphorylated in diabetic conditions. There are potential protein kinase C (PKC) binding sites predicted when the human Cx31.1 sequence is analysed via NetPhos at positions 181, 210, 227, 234, 253, and 271 (NetPhos 3.1; Technical University of Denmark). It is unclear, however, what effects this would have if Cx31.1 forms noncommunicative junctions. Increased phosphorylation can enhance stability, or reduce attrition from the membrane (Johnstone, Billaud, Lohman, Taddeo, & Isakson, 2012), so maintaining Cx31.1 membrane expression may be the main effect.

UV-triggered apoptosis in HaCaT cells increased Cx31.1 expression in cells which had been pretreated with both NGI and HGI conditions (Figure 2e). UV exposure was chosen as a physiologically relevant apoptosis trigger to mimic that seen in the epidermis following high amounts of sun exposure (C. H. Lee et al., 2013). Both the 'diabetic' treatment of cells and UV exposure were similar inducer pathways stimulating Cx31.1 to form detectable membrane gap junction plaques at significant levels ($p > .05$) above cells in non-diabetic cells not exposed to UV.

Cx31.1 was modulated over the course of HaCaT scrape-wounding and healing (Figure 3a). Cx31.1 expression increased at scrape-wound edges at both 24 and 48 hr postwounding, during injury repair, and was higher in HGI than NGI conditions. This is coincidental with the expression of active caspase 3 at 24 hr (Figure 3b), which we propose represents tissue remodelling events occurring in compartments at the wound edge. Apoptosis has an important role in wound healing (Greenhalgh, 1998) by eliminating cells that have been damaged by injury, or are not part of the

proliferating and migrating cells stimulated to close the wound. Cx31.1 may assist this process. It is also possible that Cx31.1 connexons are dispersed in the membrane (so not seen with immunocytochemistry), but with injury move into gap junction plaques, to act as a channel blocker and/or apoptosis trigger. If, for example, Cx31.1 connexons dock with Cx43 connexons, resulting heterotypic channels would be nonfunctional.

The pattern of Cx31.1 changes in expression is opposite to that seen in Cx43 modulation during scrape-wound closure in human primary skin cells (Wright et al., 2009), but implies that Cx31.1 is involved in wound healing cascades. If Cx31.1 is nonfunctional, then its increase may complement the decrease of Cx43 by acting in concert to reduce wound edge GJIC, thus assisting cell migration and wound closure (Martin et al., 2014). If the role of Cx31.1 is to close down communication, then increased Cx31.1 expression concomitantly as Cx43 declines transiently fits with formation of a wound edge communication compartment (Chanson, Watanabe, O'Shaughnessy, Zoso, & Martin, 2018; Faniku et al., 2018) in which GJIC is reduced, allowing cell migration and proliferation to be stimulated.

Co-ordinating to Cx31.1 increases, during scrape-wound closure, Caspase 3/7 activity was higher in HGI than NGI conditioned media at 0 and 24 hr (Figure 4a). Caspase levels at 48 hr were similar in NGI or HGI cultures, both increased from 0 hr. Caspase enzymes are part of the execution phase of the apoptosis pathway, and so changes in caspase 3/7 indicate changes in apoptosis levels (Al-Mashat et al., 2006). This fits with the observations that apoptosis levels in diabetic skin are at an increased level to that seen in normal skin, including wound margins (Al-Mashat et al., 2006).

In HaCaTs, which were not wounded, cells showed different responses to UV exposure, with cell viability (Figure 4b) and apoptosis levels (Figure 4c) being less affected by HGI pretreatment than those in injured cells following scrape-wounding. It may be that intact cells can withstand UV insult better, or that since Caspase 3/7 is a late apoptosis marker, the apoptosis induction window was missed. Earlier markers of apoptosis could be examined in the future, such as Annexin V. Despite the increased Cx31.1 seen with HGI treatment, HGI preconditioning may have a UV protective effect unrelated to Cx31.1 expression, likely to be associated with higher insulin levels stimulating mitogenesis (Draznin, 2011).

Studies of Cx31.1 in the skin, and other systems, suggest that it is associated with tissue turnover as well as apoptosis. This correlation has been shown in human epidermal keratinocytes (Gibson et al., 1997). and in oral keratinocytes. Cx31.1 was found to be responsive to retinoic acid (Hatakeyama, Mikami, Habano, & Takeda, 2011), where reduced Cx31.1 was thought to be due to terminal differentiation inhibition. Additionally, in small cell lung cancer models, Cx31.1 expression is regulated by autophagy (Zhu et al., 2015).

Cx31.1 has been examined in reproductive tissues, revealing potential roles in development. Cx31.1 is expressed in the placenta (Kibschull, Colaco, Matysiak-Zablocki, Winterhager, & Lye, 2014). Here, in trophoblast cells, Cx31.1 promotes placental terminal differentiation, with Cx31 knock-out delaying terminal differentiation. A lack of Cx31.1 also impaired placental development in a further study (Zheng-Fischhofer et al., 2007); placenta is a tissue that undergoes rapid tissue turnover. Furthermore, a recent study has shown that placental Cx31.1 expression is reduced in expression by high fat diet (Connor et al., 2020). In the testes, Cx31.1 is expressed in the epididymis where epithelial differentiation alters Cx31.1 expression profiles (Kidder & Cyr, 2016; Lee, 2016), as does oestradiol administration (Lee, 2017). Cx31.1 expression in the epididymis is also developmentally regulated during foetal growth (Lee & Kim, 2018).

Many studies show a clear correlation between Cx31.1 and apoptosis, such as that by Chang et al. (2009) in the eye (Chang et al., 2009) where Cx31.1 is expressed by outer epithelial cells that are about to sloughed off, and epithelial thickening occurred when Cx31.1 antisense was used to lower its expression (so those cells remained in the epithelium even as new layers were added at the bottom). In the skin, Cx31.1 is expressed continuously in the stratum granulosum and stratum spinosum layers (Aasen & Kelsell, 2009; Coutinho, Qiu, Frank, Tamber, & Becker, 2003; Zheng-Fischhofer et al., 2007). Cx31.1 expression in the upper layers of the epidermis could relate to the differentiation programme of keratinocytes at different cellular levels as they move up through the epidermal cycle and then detach during sloughing off (Aasen & Kelsell, 2009). In areas of tissue undergoing apoptosis, there is thought to be an insult threshold, when cells tip from repairing cell damage to entering into a death pathway, depending on the extent of the insult. Cells may switch on Cx31.1 at this tipping point. This was seen in ovarian follicles where the granulosa compartments of dying follicles all expressed Cx31.1 in a coordinated manner, and stopped expressing Cx43 (Wright et al., 2001). The point at which this occurs will depend on stimulus strength and there could be coordinated or uncoordinated responses depending on tissue and environment. The switch on of Cx31.1 may help the cell isolate itself from surrounding cells by closing down GJIC. Thus the health of other cells in the tissue would be preserved.

The purpose of Cx31.1 functioning to block cell–cell communication may be to act in concert with other connexins which downregulate to perform similar actions. Connexins have a short half-life (a matter of hours) and are constantly turning over. It is possible that as cells lose communication, the connexin turnover also slows down to preserve gap junctions or hemichannels that are still active. So, if a cell wants to isolate quickly, it could increase production of nonfunctional Cx31.1 to prevent any residual GJIC. Musil, Le, VanSlyke, and Roberts (2000) observed differing rates of Cx43 turnover (Musil et al., 2000) and we have also seen reductions in communication with connexin mimetic peptides as well as full prevention (Wright et al., 2009, 2012). This suggests cells producing specific "cell death connexins" would be feasible, and cells under stress in diabetic conditions would be expected to produce more Cx31.1 than those in more viable circumstances.

We conclude that Cx31.1 is modulated in this epidermal wound healing model under ‘diabetic’ conditions. Cx31.1 appears to correlate with apoptosis in the HaCaT cells, and as apoptosis is raised in

diabetic skin, it is possible that Cx31.1 expression would be modulated in wound edges during diabetic (and normal) wound healing. Future studies will confirm any potential association of Cx31.1 with tissue turn-over in diabetic wounds.

ACKNOWLEDGEMENTS

The authors thank Iain Robertson and Narges Elgaseai (Glasgow Caledonian University) for assisting with some confirmatory experiments. All authors have read and approved the final manuscript. The research was part funded by a Vacation Scholarship from Medical Research Scotland (Vac-704-2013)) to Louise Nugent and Catherine S. Wright.

CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

AUTHOR CONTRIBUTIONS

L. N. and B. O.-F. performed the research; P. E. M. and C. R. G. analysed the data and edited the paper; C. S. W. designed the study, performed the research, analysed the data, and wrote and edited the paper.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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How to cite this article: Nugent L, Ofori-Frimpong B, Martin PE, Green CR, Wright CS. Cx31.1 expression is modulated in HaCaT cells exposed to UV-induced damage and scrape-wounding. *J Cell Physiol*. 2020;1–10. https://doi.org/10.1002/jcp.29901